Kidney International, Vol. 39 (1991), pp. 591–597

LABORATORY INVESTIGATION

Effects of extracellular sodium on cytosolic calcium, PGE_2 and cAMP in papillary collecting tubule cells

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Effects of extracellular sodium on cytosolic calcium, PGE_2, and cAMP in papillary collecting tubule cells. An increase in cytosolic calcium (Ca_i^{2+}), induced by an increase in extracellular calcium concentration or addition of the calcium ionophore A23187, has been shown to suppress basal and AVP responsive cAMP and inhibit water flow in the collecting tubule. In the present study, the relationships between extracellular Na^+ concentration, Ca_i^{2+}, PGE_2 and AVP-responsive cAMP production were examined in cultured rat papillary collecting tubule (RPCT) cells. Reducing extracellular Na^+ concentration from 144 to 24 mM increased Ca_i^{2+} and PGE_2 production approximately threefold in RPCT cells. The effect of reducing media NaCl concentration below 144 mM on Ca_i^{2+} or PGE_2 was not due to a change in media osmolality or chloride concentration, since these parameters were maintained at constant levels by addition of tetramethylammonium chloride (TMA) or choline chloride. Exposure of RPCT cells to media containing 24 mM Na^+ significantly suppressed basal and AVP responsive cAMP compared to that observed at 144 mM Na^+. This suppression was mimicked by the calcium ionophore A23187 which also increased Ca_i^{2+} and PGE_2. The increase in Ca_i^{2+} and PGE_2 and the suppression of basal and AVP responsive cAMP, which were observed at 24 versus 144 mM Na^+, were abolished in calcium free media and were likely due to influx of extracellular calcium. Indomethacin did not prevent the suppressive effects of reducing extracellular Na^+ concentration below 144 mM on basal or AVP responsive cAMP, suggesting that the enhanced production of PGE_2 did not mediate the reduction in AVP responsiveness. In contrast to cAMP, reductions in extracellular Na^+ concentration from 144 to 24 mM did not influence basal cGMP or the cGMP responses to atrial natriuretic peptide or nitroprusside. In contrast to the effects of a reduction of extracellular Na^+ below 144 mM, an increase in extracellular Na^+ from 144 to 294 mM, a range of concentrations of Na^+ which is encountered under physiologic conditions in the medullary interstitium, was associated with modest reductions in Ca_i^{2+} and PGE_2, and no change in basal or AVP-responsive cAMP. These effects were due to an increase in osmolality since they were mimicked by addition of equiosmolar TMA or choline chloride. The suppression of PGE_2 production seen in RPCT cells exposed to hyperosmolar media is associated with, and may be mediated by a reduction in Ca_i^{2+}. The reduction in local PGE_2 generation by hyperosmolality may serve to enhance AVP-stimulated water absorption during hydropenia.

Numerous previous studies of mammalian collecting tubule [1–9] and toad bladder epithelium [10, 11] have supported a key role for calcium in the modulation of the hydroosmotic response to arginine vasopressin (AVP) at the sites of cAMP generation [3, 4, 7, 8] and metabolism [3, 8], as well as at post-cAMP sites [6, 12]. However, the precise relationship among changes in extracellular calcium, changes in cytosolic calcium (Ca_i^{2+}) and alterations in cAMP generation and action is not known. Studies conducted with renal papillary collecting tubule (RPCT) cells in culture [4] and in microdissected collecting tubules [3, 8, 9] and toad bladder [10] have demonstrated suppression of basal and AVP-stimulated cAMP production by the calcium ionophore A23187 or by raising media Ca^{2+} above 6.8 mM [10, 13], conditions which increase Ca_i^{2+}. By contrast, alterations in extracellular calcium from 0.5 to 4 mM, a concentration of serum calcium which may be encountered clinically in hypercalcemic states, had no effect in vitro on basal or AVP induced increases in cAMP in RPCT cells [4, 13].

Time-dependent reductions in renal medullary interstitial Na^+ concentration occur in experimental hypercalcemia in vivo [14, 15] and are thought to play a key role in the concentrating defect. In addition to the direct impact of reduced medullary solute content to impair urinary concentration, studies conducted in vitro suggest that alterations in extracellular Na^+ concentrations may influence Ca_i^{2+} by altering Na^+-Ca^{2+} exchange [16]. An increase in Ca_i^{2+} may lead to suppression of AVP responses at pre- [3, 4, 7, 8, 11] or post-cAMP sites [6, 11, 12] as well as through alterations in prostaglandin (PG) E_2 synthesis [11, 17]. The influence of extracellular Na^+ concentration on Ca_i^{2+} in RPCT cells has not previously been investigated.

In the present study we examined in RPCT cells, the influence of extracellular Na^+ concentrations from 0 to 444 mM on Ca_i^{2+}, PGE_2 production and basal and AVP induced increases in cAMP. The results support a role for Ca_i^{2+} in modulating basal PGE_2 and cAMP generation, as well as AVP responsive cAMP production in RPCT cells. By contrast, although changes in Ca_i^{2+} are often associated with changes in cGMP [18], cGMP was not altered in the present study as a function of the changes in Ca_i^{2+} induced in response to changes in extracellular Na^+ concentration. The major alterations in Ca_i^{2+}, PGE_2 and cAMP were observed when extracellular Na^+ was varied between 144 and 24 mM. Accordingly, the results do not support a major role for Na^+ induced changes in Ca_i^{2+} in the mediation of reduced concentrating ability at the Na^+ concentrations which are likely to exist in the medullary interstitium during hypercalcemia [14, 15]. However, the results do suggest that the relatively modest decrease in PGE_2 production which occurs when extracellular Na^+ is increased from 144 to 294 mM may be mediated by a fall

Received for publication March 26, 1990
and in revised form November 13, 1990
Accepted for publication November 13, 1990

in Ca_i^{2+}, and may serve to enhance concentrating ability during hydropenia.

Methods

Culture of renal papillary collecting tubule epithelial (RPCT) cells

RPCT cells were prepared as previously described [19]. The inner medullary tissue from two to three female Sprague-Dawley rats (Zivic-Miller, Pittsburgh, Pennsylvania, USA) was used to prepare enough cells for a single 24 well plate. The medullae were removed aseptically and minced finely to a past with two scalpel blades. The paste was mixed with 4 ml of type II collagenase (1 mg/ml; Cooper Biochemicals) plus 100 U/ml penicillin and 100 μg/ml streptomycin in phosphate buffered saline for two hours at 37°C, with gentle shaking every 15 minutes. At the end of 30 minutes, 6 ml of sterile H_2O was added. Large pieces of tissue were allowed to settle and the supernatant centrifuged at 200 × g. The resulting cell pellet was washed with phosphate buffered saline containing 10% bovine serum albumin, resuspended in 12 ml of tissue culture media and seeded into a 24 well plate. Tissue culture medium for plating was DMEM/F-12, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS. After 24 hours the medium was changed to defined K-1 epithelial growth medium [19]. Medium was changed daily. The cells were usually confluent after three to four days. The cells exhibited typical collecting tubule epithelial cell morphology. They were uniformly polygonal in appearance with large nuclei [20]. Hemicysts grew in cultures whose media was not changed during growth as previously described [20]. To further characterize the cells they were grown on glass coverslips, quick frozen and histochemical staining for succinate dehydrogenase, NADH diaphorase and αglycerophosphate dehydrogenase performed [21]. The RPCT cultures stained positively for NADH diaphorase and αglycerophosphate dehydrogenase but not for succinate dehydrogenase. This pattern of staining is typical of collecting tubule epithelium [20].

Incubation of cells

RPCT cells were rinsed and incubated in Krebs-Ringer bicarbonate buffer with 1 mg/ml glucose (0.5 ml/well). The standard concentration of Na^+ is 144 mM and is composed of 24 mM $NaHCO_3$ and 120 mM NaCl. All incubations for determination of PGE_2, cAMP and cGMP were conducted with confluent cultures in 24 well plates at 37°C for the times and with test agents as indicated in the text. The gas phase was 7% CO_2, balance air. Where indicated in the text, the composition of the incubation medium was changed as follows: KRBG, O Na^+ contained 120 mM tetramethylammonium chloride (TMA) and 24 mM choline bicarbonate. KRBG, 24 mM Na^+ contained 24 mM $NaHCO_3$ and 120 mM TMA. KRBG, 84 mM Na^+ contained 24 mM $NaHCO_3$, 60 mM NaCl and 60 mM TMA. KRBG, 294 mM Na^+ and KRBG, 444 mM Na^+ contained an additional 150 and 300 mM NaCl, respectively, compared to KRBG, 144 mM Na. The final osmolality of the 0, 24, 84 and 144 mM Na^+, KRBG was 305 mOsm. The final osmolalities of the 294 mM and 444 mM Na^+ media were 605 and 905, respectively. In some studies, 150 mM TMA was added to KRBG, 144 mM Na^+ to give a final osmolality of 605.

Measurement of Ca_i^{2+}

RPCT cells were grown to confluence in a 75 sq cm tissue culture flask. Cold 0.8% trypsin (4 ml) in phosphate buffered saline was added and the trypsin solution removed after 10 seconds. The cells were incubated 10 to 15 minutes at 37°C and washed off the flask with 15 ml of culture media. The cells were washed twice in 15 ml of media by centrifugation at 2000 rpm for one minute prior to loading with aequorin. The procedure for loading aequorin [22] and for measuring Ca_i^{2+} [23] were as previously described. Aequorin was purchased from Dr. J.R. Blinks (Mayo Foundation, Rochester, Minnesota, USA). RPCT cells, which had been incorporated into agarose threads [22, 23], were suspended in an aequorin photometer and perfused with KRBG, 95% O_2, 5% CO_2 at 1 ml/min for the times and with test agents as indicated in the text. The sample compartment was maintained at 37°C. Using this protocol, multiple determinations of Ca_i^{2+} were made with the same cell preparation under different experimental conditions. In the studies of the concentration response relationship between perfusate Na^+ and Ca_i^{2+}, media with the highest concentration of Na^+ to be studied was tested first. Cells were perfused with the test media for 15 minutes followed by 30 minutes of perfusion with KRBG, 95% O_2, 5% CO_2 before reducing perfusate Na^+ to the next lowest concentration. Reversing the order of changes in media Na^+ concentration had no effect on the level of Ca_i^{2+} measured at any given Na^+ concentration. Ca_i^{2+} was derived from a curve of the negative log of $[Ca^{2+}]$ in standard solutions versus the negative log of the observed fractional luminescence. Fractional luminescence was determined by dividing the observed luminescence by the maximal luminescence obtained when cells were perfused with 1% Triton X-100 [23].

Determination of PGE_2, cAMP and cGMP

PGE_2 content of the incubation media was determined by radioimmunoassay (DuPont, NEN, Boston, Massachusetts, USA) as previously described [24]. cAMP and cGMP content of RPCT cells was extracted by the addition of 0.5 ml of boiling water to the well. Media and cell extracts were combined for determination of cAMP and cGMP by radioimmunoassay (DuPont).

Statistics

Analysis of variance (ANOVA) was employed to test for differences among groups. If differences were significant by ANOVA, the significance of differences between any two mean values was determined by Student's independent *t*-test. Experiments were conducted two or three times as indicated in the footnotes to the Tables and Figures.

Results

Figure 1 shows the effects of altering perfusate Na^+ concentration from 0 to 444 mM on Ca_i^{2+} in RPCT cells. The total osmolality, bicarbonate and chloride concentration of the media which had Na^+ concentrations between 0 and 144 mM, was maintained constant by the addition of tetramethylammonium chloride (TMA) and choline bicarbonate. The total osmolality of media with 294 and 444 mM Na^+ was increased to 605 and 905 mOsm, respectively, by addition of NaCl. The composition of the media are described in detail in the **Methods** section. As

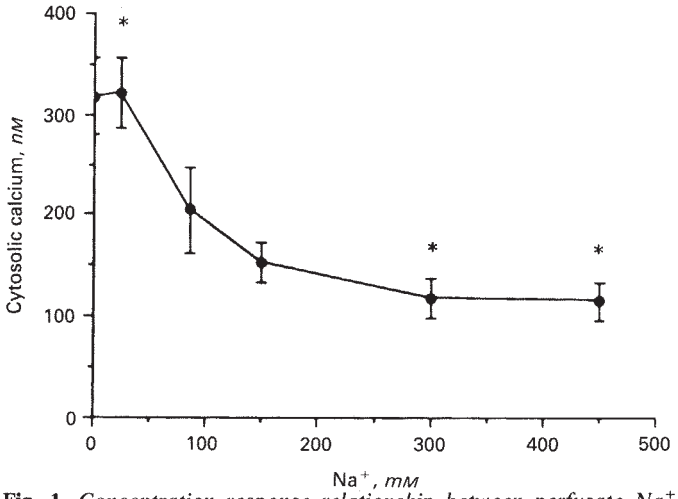

Fig. 1. *Concentration response relationship between perfusate Na^+ concentration and Ca_i^{2+} in RPCT cells.* Results shown represent means ± SE of determinations on five separate RPCT cell suspensions. All of the conditions shown were tested in each of the cell preparations. *$P <$ 0.05 compared to value for 144 mM Na^+.

illustrated in the figure, significant increases in Ca_i^{2+} were observed when media Na^+ was reduced from 144 mM to 24 mM. No further increase in Ca_i^{2+} was observed when media Na^+ was decreased from 24 mM to 0 mM. As is also shown in Figure 1, raising media Na^+ from 144 to 294 mM significantly reduced Ca_i^{2+}. No further decrease in Ca_i^{2+} was observed when media Na^+ was increased from 294 to 444 mM. In other studies, increasing media osmolality from 305 to 605 by the addition of 150 mM TMA reduced Ca_i^{2+} to the same extent as that observed by the addition of 150 mM NaCl (144 mM Na^+ 145 ± 12; 144 mM Na^+ + 150 mM TMA, 120 ± 10*; 144 mM Na^+ + 150 mM NaCl, 121 ± 10*; $P < 0.05$ vs. 144 mM Na^+).

In the studies presented in Figure 1, TMA was substituted for NaCl in order to maintain the osmolality constant at 305 mOsm, as the Na^+ concentration was reduced from 144 to 0 mM. Thus, it is possible that the increase in TMA, rather than the reduction in Na^+, may mediate the increase in Ca_i^{2+}. We consider this possibility unlikely since the addition of 150 mM TMA to media which contained 144 mM Na^+ actually reduced Ca_i^{2+} rather than increasing it. Nevertheless, to further control for the effects of TMA, we conducted additional experiments in which choline chloride was used as a substitute for NaCl, rather than TMA. The values obtained for Ca_i^{2+} when choline chloride was employed as the NaCl substitute were analogous to those obtained with TMA (24 mM Na^+ + 120 mM choline chloride, 315 ± 20*; 144 mM Na^+ 145 ± 12; 144 mM Na^+ + 150 mM choline chloride, 123 ± 10* nM Ca_i^{2+} ± SE;* $P < 0.05$ compared to 144 mM Na^+).

Figure 2A is a representative tracing demonstrating the effects of reducing perfusate Na^+ from 144 to 24 mM on Ca_i^{2+} of RPCT cells. The two minute interval between the time at which the perfusate was changed (arrow) and the time the Ca_i^{2+} starts to rise is the time it takes for the perfusate to reach the cell chamber. Ca_i^{2+} increased immediately after the low Na^+ buffer entered the cell compartment (t = 32 min) and exhibited a broad peak which lasted for about five minutes and then declined. Ca_i^{2+} had nearly, but not completely, returned to baseline by eight minutes (t = 40 min) despite continued perfusion with low Na^+ buffer (Fig. 2). Figure 2B is a tracing which illustrates the effects of exclusion of calcium from the media on basal Ca_i^{2+} and the increase in Ca_i^{2+} induced by KRBG, 24 mM Na^+. The same cell preparation was employed to obtain both tracings, and the scale is broken between 45 and 65 minutes during which time no change in Ca_i^{2+} occurred. The cells were perfused with Ca^{2+} free KRBG, 144 mM Na^+ for 15 minutes before changing the perfusate to Ca^{2+} free KRBG, 24 mM Na^+. As can be seen by comparing values for Ca_i^{2+} obtained at 45 minutes in KRBG, 144 mM Na^+, to those obtained at 65 minutes in Ca^{2+} free KRBG, 144 mM Na^+, removal of Ca^{2+} had no effect on basal Ca_i^{2+} in KRBG containing 144 mM Na^+. However, Ca^{2+}-free KRBG completely prevented the rise in Ca_i^{2+} seen when the Na^+ concentration was reduced from 144 to 24 mM. In other studies (not shown), the decrease in Ca_i^{2+} which was observed when Na^+ was increased from 144 to 294 mM persisted for at least 15 minutes. This change in Ca_i^{2+} was rapidly reversed when Na^+ was reduced from 294 to 144 mM.

Table 1 illustrates the effects of extracellular Na^+ concentration on basal PGE_2, cAMP and cGMP content of RPCT cell incubates and the effects of the calcium ionophore A23187 on each of these parameters. cGMP responses to ANF and nitroprusside, and cAMP responses to AVP are also shown. Addition of A23187 to cells incubated in KRBG, 144 mM Na^+ increased PGE_2 accumulation approximately fourfold. Exposure of cells to KRBG, 24 mM Na^+ also increased PGE_2 threefold compared to that observed in KRBG, 144 mM Na^+. Addition of A23187 to cells exposed to KRBG, 24 mM Na^+ had no further effect on PGE_2 compared to that observed in cells incubated in KRBG, 24 mM Na^+ without A23187. Exposure of RPCT to Ca^{2+}-free KRBG for 15 minutes had no effect on the basal PGE_2 production compared to that observed in complete KRBG containing 144 mM Na^+, but completely prevented the increase in PGE_2 seen when buffer Na^+ was reduced from 144 to 24 mM. As is also shown in Table 1, increasing the osmolality of the buffer from 305 to 605 with NaCl or TMA was associated with a significant reduction in PGE_2 content.

Consistent with previous studies [25], an optimally effective concentration of ANF increased cGMP content of RPCT incubates tenfold (Table 1). Nitroprusside was a relatively weak stimulus of cGMP in RPCT cells, increasing cGMP 1.5- to twofold. Addition of A23187, or altering the Na^+ content of the media had no effect on basal or stimulated cGMP under any of the conditions tested in Table 1.

As is also shown in Table 1, AVP increased cAMP of RPCT cell incubates fourfold. Consistent with previous studies [4], basal and AVP induced increases in cAMP were suppressed by the addition of A23187 to standard KRBG, 144 mM Na^+ buffer. Reducing media Na^+ from 144 to 24 mM also reduced basal and AVP responsive cAMP to an extent similar to that seen with A23187 in KRBG, 144 mM Na^+. Moreover, addition of A23187 to cells incubated in KRBG, 24 mM Na^+ did not further suppress basal or AVP induced increases in cAMP compared to that seen in KRBG, 24 mM Na^+ alone. Analogous to effects on PGE_2, incubation of RPCT cell in Ca^{2+}-free KRBG prevented suppression of basal and AVP responsive cAMP otherwise observed when extracellular Na^+ concentration was reduced. As is also shown in Table 1, increasing media osmolality from

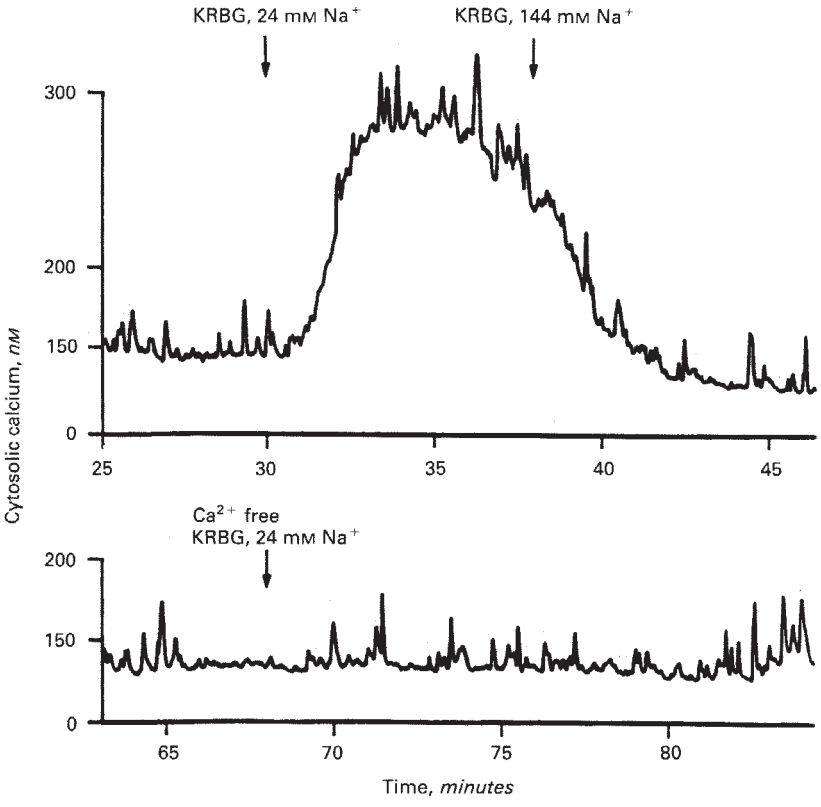

Fig. 2. *Effects of exclusion of calcium from the perfusate on increases in Ca_i^{2+} induced by a reduction in perfusate Na^+ concentration in RPCT cells.* RPCT cells were loaded with aequorin and perfused for 30 minutes with KRBG, 144 mM Na^+. The perfusate was then changed sequentially, to KRBG, 24 mM Na^+ (30 to 38 min), KRBG, 144 mM Na^+ (38 to 53 min), Ca^{2+} free KRBG, 144 mM Na^+ (53 to 68 min) and Ca^{2+} free KRBG, 24 mM Na^+ (68 to 80 min) as indicated by the arrows. Results shown are from a single representative experiment repeated twice.

Table 1. Effects of media sodium concentration on basal and hormone induced increases in PGE_2, cGMP and cAMP content of RPCT cells

Incubation conditions	Total osmolality *mOsm*	PGE_2 *ng/mg protein*	cGMP *pmol/mg protein*			cAMP *pmol/mg protein*	
			—	ANF	Nitro-prusside protein	—	AVP
KRBG, 144 mM Na^+	305	6.6 ± 0.5	3.2 ± 0.3	34 ± 3	4.9 ± 0.4	50 ± 7	206 ± 18
+ 10 μM A23187	305	25[a] ± 3	2.9 ± 0.4	37 ± 4	5.1 ± 0.6	22[a] ± 2	27[a] ± 4
KRBG, 24 mM Na^+ + 120 mM TMA	305	18[a] ± 2	2.7 ± 0.3	39 ± 5	4.8 ± 0.5	28[a] ± 4	34[a] ± 4
+ 10 μM A23187	305	23[a] ± 2	2.8 ± 0.3	33 ± 3	4.7 ± 0.7	26[a] ± 5	29[a] ± 5
Ca^{2+} free KRBG, 144 mM Na^+	305	6.4 ± 0.6	3.1 ± 0.4	36 ± 3	5.3 ± 0.4	52 ± 6	228 ± 38
Ca^{2+} free KRBG, 24 mM Na^+ + 120 mM TMA	305	6.4[b] ± 0.5	2.9 ± 0.2	35 ± 4	4.9 ± 0.7	49[b] ± 5	212[b] ± 27
KRBG 294 mM Na^+	605	4.8[a] ± 0.7	3.1 ± 0.5	39 ± 3	5.1 ± 0.4	45 ± 7	194 ± 24
KRBG 144 mM Na^+ + 150 mM TMA	605	4.7[a] ± 0.8	2.8 ± 0.3	36 ± 5	4.6 ± 0.5	42 ± 7	192 ± 16

RPCT cells were pre-incubated for 15 minutes in KRBG. The media was then changed to that indicated under incubation conditions and the incubation continued for 15 minutes. Where indicated ANF (10^{-7}M), sodium nitroprusside (10^{-5}M) or AVP (10^{-7}M) was present for 15 minutes during the second incubation period. 3-Isobutyl 1-methylxanthine (MIX, 0.5 mM) was present in incubations employed for determination of cAMP and cGMP. Separate incubations were used for the determination of PGE_2. PGE_2 content of the second incubation media is shown. cAMP and cGMP content of the media plus cell extracts is shown. Results represent means ± SE of triplicate incubations from each of three separate experiments.

[a] $P < 0.05$ compared to KRBG, 144 mM Na^+

[b] $P < 0.05$ comparing Ca^{2+} free KRBG, 24 mM Na^+ plus 120 mM TMA to KRBG, 24 mM Na^+ plus 120 mM TMA

305 to 605 with TMA or NaCl had no effect on basal or AVP induced increases in cAMP compared to that observed in KRBG, 144 mM Na^+.

As shown in Table 2, indomethacin had no effect on the suppression of basal and AVP responsive cAMP induced by incubation of RPCT cells in KRBG, 24 mM Na^+. As is also shown, this concentration of indomethacin reduced PGE_2 accumulation in RPCT cell incubates by greater than 80% and abolished increases in PGE_2 induced by reducing extracellular Na^+ from 144 to 24 mM.

Discussion

Results of the present study demonstrate that decreasing perfusate Na^+ from 144 to 24 mM increases Ca_i^{2+} approximately threefold, possibly by increasing Na^+-Ca^{2+} exchange in the reverse mode [16]. The effect of reduced extracellular Na^+

Table 2. Effects of indomethacin on PGE_2 and basal and AVP induced increases in cAMP of RPCT cells exposed to low sodium buffer

Incubation conditions	cAMP *pmol/mg protein*		PGE_2 *ng/mg protein*
	—	AVP	
KRBG, 144 mM Na^+	47 ± 6	256 ± 28	6.2 ± 0.3
KRBG, 24 mM Na^+ + 120 mM TMA	28[a] ± 3	31[a] ± 5	16[a] ± 3
+ Indomethacin, 25 μM	29[a] ± 4	35[a] ± 3	2.6[a] ± 0.2

Studies were conducted as described in the footnote to Table 1. Where indicated, indomethacin was present for 15 minutes during the second incubation period. Results shown are means ± SE of triplicate determinations from each of two separate experiments.

[a] $P < 0.05$ compared to KRBG, 144 mM Na^+

concentration was not related to a change in extracellular osmolality or chloride since osmolality and chloride were maintained constant in the reduced Na^+ media by the addition of TMA. A previous study demonstrated that decreasing extracellular Na^+ triggers release of Ca_i^{2+} from intracellular pools in cultured fibroblasts [26]. However, the increase in Ca_i^{2+} observed at 24 versus 144 mM Na^+ in the present study was abolished by exclusion of perfusate calcium, and was likely due to influx of extracellular calcium, similar to the findings in monkey kidney cells [16]. Increasing Na^+ from 144 to 294 mM suppressed Ca_i^{2+}. However, the effects of raising media Na^+ on Ca_i^{2+} were likely due to an increase in total osmolality per se, since raising media osmolality from 305 to 605 mOsm by addition of TMA mimicked the effects of addition of NaCl on Ca_i^{2+}.

Previous studies have demonstrated that A23187 reduces basal and AVP induced increases in cAMP in cultured RPCT cells [4], microdissected medullary and papillary collecting tubules [3, 8], and medullary thick ascending limbs [5], an effect which is likely mediated by an increase in Ca_i^{2+}. The effect of A23187 on AVP stimulated cAMP of microdissected collecting tubules was more pronounced in the absence than in the presence of a phosphodiesterase inhibitor [3, 8], suggesting that A23187 was both inhibiting cAMP generation and increasing cAMP metabolism. In the present study the phosphodiesterase inhibitor, 3-isobutyl 1-methyl xanthine (MIX), was employed in all incubations for determination of cyclic nucleotides. Thus, the observed effects of altered media Na^+ concentration on cAMP reflect predominantly altered cAMP generation. In this regard, results of the present study which demonstrate that reducing media Na^+ from 144 to 24 mM increased Ca_i^{2+} and reduced basal and AVP responsive cAMP support a suppressive effect of raising Ca_i^{2+} on cAMP generation.

By contrast, consistent with a previous study [27], we found no effect of raising extracellular Na^+ from 144 to 294 mM, corresponding to an increase in osmolality from 305 to 605 mOsm, on basal or AVP responsive cAMP when assessed in the presence of MIX, despite the fact that Ca_i^{2+} and PGE_2 production were reduced in cells incubated at the higher Na^+ concentration. Of interest, the cAMP response to AVP was previously reported to be enhanced in RPCT cells incubated in the absence, but not in the presence of a phosphodiesterase inhibitor when the osmolality of the incubation medium was raised from 305 to 750 mOsm with NaCl [27], implying an action of hyperosmolar Na^+ at the level of cAMP metabolism rather than synthesis.

Biphasic effects of changes in extracellular calcium concentration (0 to 8 mM) on AVP responsive cAMP in RPCT cells have been reported [7, 13]. However, when extracellular calcium was varied within a clinically relevant range (0.5 to 4 mM), no effects on Ca_i^{2+} or cAMP were observed [4, 7, 13]. In the present study we found no effect of a brief 15 minute exposure of RPCT cells to Ca^{2+} free perfusate without EGTA on Ca_i^{2+} or on basal or AVP responsive cAMP. Our results are not necessarily inconsistent with those in the earlier study. In that report, RPCT cells were exposed to Ca^{2+} free buffer plus EGTA for one hour to reduce Ca_i^{2+} to 25 nM and to reduce AVP responsive cAMP. The present results demonstrate that an increase in Ca_i^{2+} from 150 to 315 nM is sufficient to abolish the cAMP response to AVP. This finding is entirely consistent with results previously reported in RPCT cells exposed to 6.2 mM Ca^{2+} [13].

Studies from our own [24] and other [28, 29] laboratories have demonstrated that increasing media osmolality with NaCl stimulates PGE_2 production in rat inner medullary slices [24, 28] and microdissected papillary collecting ducts [29]. Nevertheless, in RPCT cells, reduced PGE_2 production was observed when media osmolality was raised by addition of NaCl. Sato and Dunn reported a similar response in these cells [27]. The basis for the differences between results obtained in inner medullary slices and microdissected tubules compared to RPCT cells are not apparent.

It is well recognized that renal prostaglandins antagonize AVP action in vivo [30, 31] and in vitro [32]. However, the mechanism by which this occurs is not known. Most previous studies have failed to find suppressive effects of low concentrations of exogenous PGE_2 on AVP stimulated cAMP production in RPCT cells [33–35]. However, suppression of AVP responsive cAMP by exogenous PGE_2 was observed when cells were incubated in hypertonic (1800 mOsm) but not isotonic media [36]. Increasing endogenous PGE_2 production by addition of A23187 [3, 4] to either RPCT cells in culture [4] or to microdissected medullary tubules [3] was associated with suppression of AVP stimulated cAMP. Nevertheless, inhibitors of cyclooxygenase activity did not prevent the effects of A23187. These studies support the notion that an increase in Ca_i^{2+} may exert an inhibitory effect on AVP responsive cAMP which is independent of enhanced prostaglandin production. The results of the present study, which demonstrate that indomethacin does not prevent the fall in basal and AVP responsive cAMP which occurs with a reduction in extracellular Na^+ to 24 mM, are consistent with these previous studies. The mechanism by which an increase in Ca_i^{2+} suppresses basal and AVP responsive cAMP is not known.

Studies conducted in toad urinary bladder have demonstrated suppression of AVP action on urea permeability under conditions of reduced serosal Na^+ concentration [11]. In these studies, two separate effects of reduced Na^+ were observed. One was dependent on the presence of serosal calcium and altered pre- and post-cAMP sites. The second was independent of serosal calcium and altered a pre-cAMP site. The Ca^{2+}-dependent suppression of AVP action in toad bladder, seen at serosal Na^+ concentrations between 10 and 50 mM appears to

be analogous to that observed in RPCT cells. Nevertheless, in contrast to results obtained in RPCT cells, indomethacin partially suppressed the inhibitory effect of low media Na^+ on AVP induced increases urea permeability in toad bladder [11], supporting a role for cyclooxygenase products in the mediation of at least a portion of this effect in toad bladder.

Previous studies have suggested that changes in Ca_i^{2+} may regulate net sodium transport by inhibiting the passive diffusion of Na^+ into the cell [37]. ANF inhibits Na^+ uptake into the cell and this process is mediated by cGMP [38, 39]. Since increases in Ca_i^{2+} are often accompanied by increases in cGMP [18], and an increase in cGMP may suppress passive Na^+ uptake, we examined the possibility that the increase in Ca_i^{2+} which occurred when extracellular Na^+ was reduced from 144 to 24 mM might increase cGMP or enhance cGMP responses to ANF. RPCT cells responded to ANF with tenfold increase in cGMP, but were far less responsive to nitroprusside (1.5-fold). Media Na^+ concentrations from 24 to 294 mM had no effect on basal, ANF or nitroprusside responsive cGMP compared to that observed at 144 mM Na^+. Moreover, A23187 was also without effect on cGMP, suggesting that the guanylate cyclase of RPCT cells was relatively unresponsive to changes in Ca_i^{2+}. At least three forms of guanylate cyclase have been described: a plasma membrane form which contains the ANF receptor; a cytosolic form which is responsive to NO and N-nitroso compounds; and a Ca^{2+} responsive form which is present in the cytoskeleton and plasma membrane [40]. Results of the present study suggest that guanylate cyclase of RPCT cells is primarily the plasma membrane form which responds to ANF. In this regard, we have previously demonstrated that rat inner medullary guanylate cyclase is found predominantly in the particulate fraction of cell homogenates [41].

The present observations may have physiologic relevance, but they must be interpreted with caution. Hypercalcemia is accompanied by a reduction in medullary interstitial Na^+ concentration which in turn is thought to contribute to the renal concentrating defect [14, 15], presumably through a direct hydrosmotic effect. However, interstitial papillary Na^+ concentrations probably do not fall below that of plasma [14, 15]. In the present study, relatively minor effects of reductions in extracellular Na^+ from 294 and 144 mM were observed on Ca_i^{2+} and PGE_2, and changing extracellular Na^+ concentrations over this range did not influence basal or AVP responsive cAMP. Accordingly, it seems unlikely that the alterations in Ca_i^{2+} or PGE_2 production which occur in RPCT cells exposed to a range of concentrations of Na^+ found in the medullary interstitium play a major role in the concentrating defect associated with hypercalcemia. Whether these effects of reduced medullary Na^+ concentration participate in dampening the hydroosmotic action of AVP is uncertain. It is possible that even the small changes in PGE_2 observed might influence the hydroosmotic actions of AVP through cAMP independent mechanisms, such as through alterations in vasa recta blood flow or solute absorption in the medullary collecting tubule or thick ascending limb [27]. Conversely, the fall in Ca_i^{2+} and local PGE_2 generation which occurs when extracellular Na^+ concentration is increased during hydropenia may enhance the hydroosmotic response of RPCT cells to AVP.

Acknowledgments

This work was supported by the General Medical Research Service of the Veterans Administration. The authors are indebted to Karen Thornburg for technical assistance and to Florence Flick for secretarial support. We are also grateful to Drs. Andre Borle and Rebecca K. Studer, Department of Physiology, University of Pittsburgh, for assistance in performing the measurements of Ca_i^{2+}.

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References

1. Goldfarb S: Effects of calcium on ADH action in the cortical collecting tubule perfused *in vitro*. *Am J Physiol* F481–F486, 1982
2. Ishikawa S, Saito T, Kuzuya T: Calmodulin regulation of cellular cyclic AMP production in response to arginine vasopressin, prostaglandin E_2 and forskolin in rat renal papillary collecting tubule cells in culture. *J Endocrinol* 107:15–22, 1985
3. Kusano E, Murayama N, Werness JL, Christensen S, Homma S, Yusufi ANK, Dousa TP: Effects of calcium on the vasopressin-sensitive cAMP metabolism in medullary tubules. *Am J Physiol* 249:F956–F966, 1985
4. Teitelbaum I, Berl T: Effects of calcium on vasopressin-mediated cyclic adenosine monophosphate formation in cultured rat inner medullary collecting tubule cells, evidence for the role of intracellular calcium. *J Clin Invest* 77:1574–1583, 1986
5. Takaichi K, Uchida S, Kurokawa K: High Ca^{2+} inhibits AVP-dependent cAMP production in thick ascending limbs of Henle. *Am J Physiol* 250:F770–F776, 1986
6. Dillingham MA, Dixon BS, Anderson RJ: Calcium modulates vasopressin effect in rabbit cortical collecting tubule. *Am J Physiol* 252:F115–F121, 1987
7. Ishikawa SE, Okada K, Saito T: Arginine vasopressin increases cellular free calcium concentration and adenosine 3'5'monophosphate production in rat renal papillary collecting tubule cells in culture. *Endocrinology* 123:1376–1384, 1988
8. Jackson BA: Modulation of vasopressin sensitive cyclic AMP levels by calcium in papillary collecting tubules. *Molec Cell Endo* 57:199–204, 1988
9. Ando Y, Jacobson HR, Breyer MD: Phorbol Ester and A23187 have additive but mechanistically separate effects on vasopressin action in rabbit collecting tubule. *J Clin Invest* 81:1578–1584, 1988
10. Omachi RS, Robbie DE, Handler JS, Orloff J: Effects of ADH and other agents on cyclic AMP accumulation in toad bladder epithelium. *Am J Physiol* 226:1152–1157, 1974
11. Hardy MA, Ware HM: Roles of Ca^{2+} and Na^+ on the modulation of antidiuretic hormone action on urea permeability in toad urinary bladder. *J Clin Invest* 75:921–931, 1985
12. Frindt G, Windhager EE, Taylor A: Hydroosmotic response of collecting tubules to ADH or cAMP at reduced peritubular sodium. *Am J Physiol* 243:F503–F512, 1982
13. Ishikawa SE, Saito T: Optimal concentration of cellular free calcium for AVP-induced cAMP in collecting tubules. *Kidney Int* 37:1060–1066, 1990
14. Levi M, Peterson L, Berl T: Mechanism of concentrating defect in hypercalcemia, role of polydipsia and prostaglandins. *Kidney Int* 23:489–497, 1983
15. Manitius A, Levitin H, Beck D, Epstein FH: On the mechanism of impairment of renal concentration ability in hypercalcemia. *J Clin Invest* 39:693–697, 1960
16. Snowdowne KW, Borle AB: Effects of low extracellular sodium on cytosolic ionized calcium: Na^+-Ca^{2+} exchange as a major calcium influx pathway in kidney cells. *J Biol Chem* 260:14998–15007, 1985
17. Beck TR, Dunn MJ: The relationship of antidiuretic hormone and renal prostaglandins. *Miner Electrol Metab* 6:46–59, 1981
18. Craven PA, DeRubertis FR: Calcium and O_2-dependent control of inner medullary cGMP: Possible role for Ca^{2+}-dependent arachidonate release and prostaglandin synthesis in expression of the

action of osmolality on renal inner medullary guanosine 3'5'monophosphate. *Metabolism* 29:842–853, 1980
19. Reid GM, Appel RG, Dunn MJ: Papillary collecting tubule synthesis of prostaglandin E_2 on Dahl rats. *Hypertension* 11:179–184, 1988
20. Grenier FC, Rollins TE, Smith WL: Kinin induced prostaglandin synthesis by renal papillary collecting tubule cells in culture. *Am J Physiol* 241:F94–F104, 1981
21. Nagle RB, Bulger RE, Cutler RE, Jervis HR, Benditt EP: Unilateral obstructive nephropathy in the rabbit I: Early morphologic, physiologic and histochemical changes. *Lab Invest* 28:546–567, 1973
22. Borle AB, Freudenrich CC, Snowdowne KW: A simple method for incorporating aequorin into mammalian cells. *Am J Physiol* 251:C323–C326, 1986
23. Snowdowne KW, Borle AB: Measurement of cytosolic free calcium in mammalian cells with aequorin. *Am J Physiol* 247:C396–C408, 1984
24. Craven PA, Briggs R, DeRubertis FR: Calcium dependent action of osmolality and adenosine 3'5'monophosphate accumulation in rat inner medulla: Evidence for a relationship to calcium responsive arachidonate release and prostaglandin synthesis. *J Clin Invest* 65:529–542, 1980
25. Nonoguchi H, Knepper MA, Manganiello VC: Effects of atrial natriuretic factor on cyclic guanosine monophosphate and cyclic adenosine monophosphate accumulation in microdissected nephron segments from rats. *J Clin Invest* 79:500–507, 1987
26. Smith JB, Dwyer SD, Smith L: Decreasing extracellular Na^+ concentration triggers inositol polyphosphate production and Ca^{2+} mobilization. *J Biol Chem* 264:831–837, 1989
27. Sato M, Dunn MJ: Osmolality vassopressin-stimulated cAMP and PGE_2 synthesis in rat collecting tubule cells. *Am J Physiol* 250: F802–F810, 1986
28. Dannon A, Knapp HR, Oelz O, Oates JA: Stimulation of prostaglandin biosynthesis in the renal papilla by hypertonic mediums. *Am J Physiol* 234:F64–F67, 1978
29. Jackson BA: Prostaglandin E_2 synthesis in the inner medullary collecting duct of the rat: Implications for vasopressin-dependent cyclic AMP formation. *J Cell Physiol* 129:60–64, 1986
30. Fejes-Toth GA, Magyor A, Walter J: Renal response to vasopressin after inhibition of prostaglandin synthesis. *Am J Physiol* 232:F416–F423, 1977
31. Anderson RJ, Berl T, McDonald KM, Schrier RW: Evidence for *in vivo* antagonism between vasopressin and prostaglandin in the mammalian kidney. *J Clin Invest* 56:420–426, 1975
32. Grantham JJ, Orloff J: Effect of prostaglandin E_1 on the permeability response of the isolated collecting tubule to vasopressin, adenosine 3'5'monophosphate and theophylline. *J Clin Invest* 47:1154–1161, 1968
33. Sato M, Dunn MJ: Interaction of vasopressin prostaglandin and cAMP in rat renal papillary collecting tubule cells in culture. *Am J Physiol* 247:F423–F433, 1984
34. Pugliese F, Sato M, Williams S, Aikawa M, Hassid A, Dunn M: Rabbit and rat renal papillary collecting tubule cells in culture: The interactions of arginine vasopressin, prostaglandins and cyclic AMP. *Adv Prost Thromb Leuk Res* 11:517–523, 1983
35. Grenier FC, Allen ML, Smith WL: Interrelationships among prostaglandins, vasopressin and cAMP in renal papillary collecting tubule cells in culture. *Prostaglandins* 24:547–565, 1982
36. Ishikawa SE, Saito T, Kuzuya T: Effects of osmolality on the interactions between vasopressin and prostaglandin in rat renal papillary collecting tubule cells in culture. *Biomed Res* 6:269–278, 1985
37. Taylor A, Windhager EE: Possible role of cytosolic calcium and Na-Ca exchange in regulation of transepithelial sodium transport. *Am J Physiol* 236:F505–F512, 1979
38. Zeidel ML, Seifter JL, Lear S, Brenner BM, Silva P: Atrial peptides inhibit oxygen consumption in kidney medullary collecting duct cells. *Am J Physiol* 251:F379–F383, 1986
39. Mohrmann M, Cantiello HF, Ausiello DA: Inhibition of epithelial Na^+ transport by atriopeptin, protein kinase C and pertussis toxin. *Am J Physiol* 253:F372–F376, 1987
40. Schulz S, Chinker M, Garbers DL: The guanylate cyclase receptor family of proteins. *FASEB J* 3:2026–2035, 1989
41. Craven PA, DeRubertis FR: Properties and subcellular distribution of guanylate cyclase activity in the rat renal medulla. Correlation with tissue content of guanosine 3'5'monophosphate. *Biochemistry* 15:5131–5139, 1976